# Overexpression of a plasma membrane $Na^+/H^+$ antiporter gene improves salt tolerance in *Arabidopsis thaliana*

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High concentrations of $Na^+$ in saline soils inhibit plant growth and reduce agricultural productivity. We report here that CaMV 35S promoter driven overexpression of the *Arabidopsis thaliana SOS1* gene, which encodes a plasma membrane $Na^+/H^+$ antiporter, improves plant salt tolerance in *A. thaliana*. Transgenic plants showed substantial upregulation of *SOS1* transcript levels upon NaCl treatment, suggesting post-transcriptional control of *SOS1* transcript accumulation. In response to NaCl treatment, transgenic plants overexpressing *SOS1* accumulated less $Na^+$ in the xylem transpirational stream and in the shoot. Undifferentiated callus cultures regenerated from the transgenic plants were also more tolerant of salt stress, which was correlated with reduced $Na^+$ content in the transgenic cells. These results show that improved salt tolerance could be achieved by limiting $Na^+$ accumulation in plant cells.



Soil salinity is a major factor in reducing plant growth and productivity. One strategy for improving the salt tolerance of a plant is to increase the production of small osmolytes or stress proteins that protect or reduce damage caused by salt stress[1]. This strategy was pioneered by Tarczynski *et al.*[2], who showed that transgenic tobacco plants overexpressing the bacterial *mtlD* gene produced mannitol and had enhanced salt tolerance[2]. Since then, a number of osmolytes such as ononitol[3], proline[4], glycinebetaine[5], trehalose[6], ectoine[7], and fructan[8] have been engineered in transgenic plants to improve salt tolerance or water-stress tolerance.

Overexpression of the barley *HVA1* gene, encoding a LEA/dehydrin-type stress protein, conferred salt tolerance on the transgenic rice plants[9]. The *A. thaliana* CBF/DREB proteins are a family of transcription factors that bind to the DRE/CRT *cis* element on the promoters of a number of stress genes[10,11]. Ectopic expression of these transcription factors activates the expression of downstream stress genes in the absence of stress, and improves plant tolerance to salt, drought, and freezing stress[11–13]. Several other regulatory genes, such as rice *CDPK*[14], the alfalfa zinc-finger protein gene *Alfin1* (refs. 15,16), and tobacco *NPK1* (ref. 17), have also been ectopically expressed in transgenic plants to enhance stress tolerance.

In contrast to the large number of reports on improving salt tolerance through the strategy of damage control, there have been only a few studies aimed at increasing salt tolerance by helping plants re-establish homeostasis under stress. Calcineurin and HAL1 are regulators of intracellular $K^+$ and $Na^+$ homeostasis in yeast[18,19]. Ectopic expression of these yeast regulatory proteins improves salt tolerance in transgenic plants[20,21]. Recently, overexpression of either the *A. thaliana* vacuolar $Na^+/H^+$ antiporter AtNHX1 or the vacuolar $H^+$-pyrophosphatase AVP1 was reported to confer salt tolerance on transgenic plants[22–24].

The *A. thaliana SOS1* gene encodes a plasma membrane $Na^+/H^+$ antiporter that is essential for salt tolerance[25–27]. We report here that overexpression of *SOS1* improves salt tolerance in transgenic *A. thaliana*. Increased salt tolerance in the transgenic plants is correlated with reduced $Na^+$ accumulation under salt stress. This is the first time that a $Na^+$ efflux carrier has been ectopically expressed in plants. Our results establish a novel strategy to improve salt tolerance by limiting $Na^+$ accumulation in plants.

## Results

**Increased salt tolerance conferred by *SOS1* overexpression.** *A. thaliana* plants were transformed with a construct containing the *SOS1* cDNA driven by the cauliflower mosaic virus (CaMV) 35S promoter. We obtained 29 kanamycin-resistant $T_1$ transgenic plants harboring the *35S:SOS1* transgene. In the absence of salt, all the transgenic $T_1$ lines flowered at the same time and reached expected final size, as did the control plants transformed with the vector only. Two $T_2$ transgenic plants from each $T_1$ individual transgenic line were selected and compared with the control plants in salt-tolerance tests. When watered with 0.05× Murashige-Skoog (MS) nutrient salts in water, the *35S:SOS1* $T_2$ transgenic plants showed no differences in either vegetative or reproductive growth from wild-type plants. However, during treatment with progressively increasing concentrations of NaCl, control plants displayed progressive chlorosis, growth inhibition, and decreased vigor. As a result of a general loss of vigor of the meristematic tissue, far fewer control plants bolted and produced inflorescences during the salt exposure. The transgenic plants that overexpressed *SOS1* showed better growth than the control plants during the salt treatment (Fig. 1A). Of the 58 transgenic plants carrying *35S:SOS1*, 93% bolted and set seed, whereas only 13% of the 45 control plants bolted and none set

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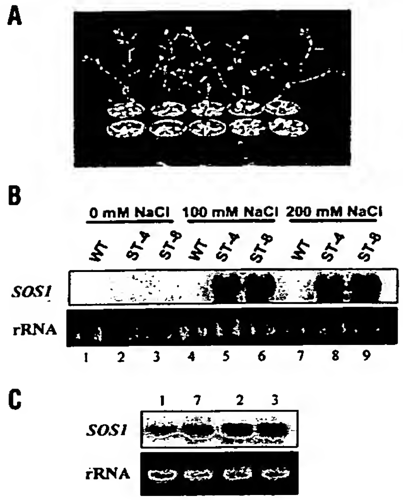


**Figure 1.** Overexpression of *SOS1* improves salt tolerance of *A. thaliana* plants. (A) 58 $T_2$ transgenic plants overexpressing *SOS1* and 45 control plants that were transformed with vector only were compared for their salt tolerance. Immediately after the whole process of treatment, representative plants were chosen and this picture taken. Front row, control plants; rear row, *35S:SOS1* transgenic plants. (B, C) Northern analysis of *SOS1* transcript levels. 1, wild-type control; 2, ST-4 control; 3, ST-8 control; 4, wild-type plants treated with 100 mM NaCl for 12 h; 5, ST-4 transgenic plants treated with 100 mM NaCl for 12 h; 6, ST-8 transgenic plants treated with 100 mM NaCl for 12 h; 7, wild-type plants treated with 200 mM NaCl for 12 h; 8, ST-4 transgenic plants treated with 200 mM NaCl for 12 h; 9, ST-8 transgenic plants treated with 200 mM NaCl for 12 h. The *SOS1* blot in (B) was exposed for 5 h and in (C) for 3 d.

seed. Out of the 58 $T_2$ *35S:SOS1* transgenic plants tested, two independent lines, designated ST-4 and ST-8, seemed to have the highest level of salt tolerance and were selected for further investigation. Homozygous $T_4$ transgenic plants were selected for subsequent tests based on the lack of segregation.

RNA blot analysis showed that the two transgenic lines had higher levels of *SOS1* transcript than wild-type plants with or without salt treatment (Fig. 1B, C). Interestingly, *SOS1* transcript levels were not very high in the transgenic plants without NaCl treatment but increased greatly upon NaCl treatment (Fig. 1B, C). The increase in transcript accumulation in transgenic plants was markedly higher than that in the control line (Fig. 1B). In the absence of NaCl or during NaCl treatments with concentrations of 200 mM, the transgenic line ST-8 displayed a slightly higher *SOS1* transcript level than the transgenic line ST-4 (Fig. 1B, C).

We tested homozygous transgenic plants from these two lines in another salt-tolerance assay. *35S:SOS1* transgenic and control plants were grown in MS agar medium plus 200 mM NaCl for 5 days and transferred to soil under normal growth conditions for three weeks. Approximately 68% of ST-4 and 76% of ST-8 transgenic plants survived and continued to grow, whereas only 28% of control plants survived (Table 1A). Both the *35S:SOS1* transgenic and control plants had a 100% survival rate when they were not treated with NaCl.

**Table 1. *SOS1* overexpression improves root growth, protein and chlorophyll content, and plant survival under salt stress**

**A. Survival rate[a]**

| | Survival | Total | % |
|---|---|---|---|
| ST-4 | 41 | 60 | 68.3 |
| ST-8 | 45 | 59 | 76.3 |
| WT:vector | 17 | 60 | 28.3[b] |

**B. Effect on root growth (cm/week)**

| | 0 mM NaCl | 100 mM NaCl | Relative growth (%) |
|---|---|---|---|
| ST-4 | 4.92 (0.31)[c] | 3.02 (0.21)[d] | 61.4 |
| ST-8 | 4.86 (0.34)[c] | 3.20 (0.25)[d] | 65.8 |
| WT:vector | 4.76 (0.27)[c] | 2.40 (0.16)[e] | 50.4 |

**C. Changes in total protein level (mg/g FW)**

| | 0 mM NaCl | 120 mM NaCl | Reduction (%) |
|---|---|---|---|
| ST-4 | 29.12 (2.31)[c] | 18.54 (0.82)[d] | 36.3 |
| ST-8 | 28.23 (2.68)[c] | 20.47 (0.95)[d] | 27.5 |
| WT:vector | 29.85 (1.90)[c] | 11.37 (0.59)[e] | 61.9 |

**D. Changes in chlorophyll content**

| | Chl a (mg/g FW) | | Chl b (mg/g FW) | | Total Chl (mg/g FW) | |
|---|---|---|---|---|---|---|
| NaCl conc. (mM) | 0 | 120 | 0 | 120 | 0 | 120 |
| ST-4 | 0.91 (0.04) | 0.49 (0.04) | 0.88 (0.08) | 0.57 (0.03) | 1.79 (0.09)[c] | 1.06 (0.08)[d] |
| ST-8 | 0.96 (0.06) | 0.57 (0.03) | 0.85 (0.06) | 0.61 (0.04) | 1.81 (0.10)[c] | 1.18 (0.05)[d] |
| WT:vector | 0.93 (0.03) | 0.32 (0.03) | 0.92 (0.11) | 0.42 (0.09) | 1.85 (0.08)[c] | 0.75 (0.12)[e] |

[a]Experiment was repeated three times. [b]Statistical significance, as compared with the value of WT:*35SSOS1*, was determined by $\chi^2$ test ($P < 0.01$). Values with different superscript letters (c–e) indicate significant difference at $P < 0.05$ (Fisher's protected LSD test). Numbers in parentheses are standard deviations ($n = 3$). FW, fresh weight.

**Increased root growth and photosynthetic capacity.** The root growth of *SOS1* transgenic and control plants was measured. Without NaCl treatment, there was no significant difference in root growth rate between transgenic and control plants (Table 1B). However, root growth of the transgenic plants was less inhibited by NaCl treatment (Table 1B). Consequently, the relative root growth rate for the *SOS1* transgenic plants was higher than that for control plants.

We used total protein content as a general indicator of plant growth and metabolism, and measured it in the transgenic plants and control plants. Upon NaCl treatment, the total protein content in control plants decreased by ~61.9%, compared with only 27.5% in ST-8 and 36.3% in ST-4 plants (Table 1C).

In response to salt-stress treatment, the quantum yield of electron-transport activity decreased in both the *SOS1*-overexpressing

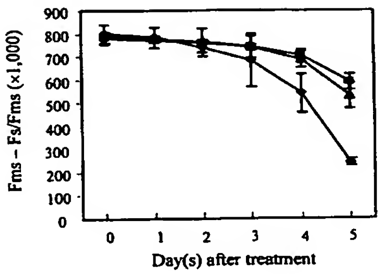


**Figure 2.** Changes of quantum yield in control and *35S:SOS1* transgenic plants. The solution for salt treatment contained 0.05× MS salts plus 150 mM NaCl. Plants were irrigated with salt solution at days 0 and 2. Diamonds, control; squares, *35S:SOS1*, line ST-8; triangle, *35S:SOS1*, line ST-4. Error bars represent s.d. ($n = 6$).

plants and control plants (Fig. 2). No significant difference in quantum yield was detected between the control and ST-4 or ST-8 plants during 3 days of NaCl treatment (Fig. 2). However, after 4 and 5 days of NaCl treatment, the quantum yield of control plants decreased dramatically, whereas only a slight decrease was detected in ST-4 and ST-8 plants (Fig. 2). Furthermore, although the chlorophyll a, b, and total chlorophyll content decreased upon salt stress in both transgenic and control plants, the extent of this decline was less in the transgenic plants (Table 1D). These results indicate that the *SOS1*-overexpressing transgenic plants may have higher light harvest and photosynthetic capacities than control plants under salt stress.

Enhanced early seedling development under salt stress. To determine whether *SOS1*-overexpressing plants have elevated salt tolerance during seed germination and early seedling development, seeds from five homozygous *SOS1*-overexpressing lines were germinated on MS media containing different levels of NaCl. On medium without NaCl, the *SOS1* transgenic plants did not show any significant difference from control plants during germination and early development (Figs. 3A, D). On medium containing 50 mM NaCl, both the control and *SOS1*-overexpressing seeds germinated, but the control seedlings showed more anthocyanin accumulation, smaller cotyledon size, and less root growth than the *SOS1*-overexpressing plants (Figs 3B, E). At a higher concentration of NaCl (150 mM) in the medium, both the control and *SOS1*-overexpressing seeds could still germinate, but the control plants were severely damaged during germination (Fig. 3C). All the cotyledons of the control plants were bleached. However, about 50% of ST-4 and ST-8 transgenic lines had green cotyledons on 150 mM NaCl (Fig. 3C).

Reduced $Na^+$ accumulation in plants overexpressing *SOS1*. Under severe salt stress, SOS1 functions to retrieve $Na^+$ from the xylem to limit accumulation of $Na^+$ in the shoot[26]. To determine if overexpression of *SOS1* reduces $Na^+$ accumulation in *A. thaliana*, the $Na^+$ content in transgenic plants overexpressing *SOS1* and control plants was examined. In response to 100 mM NaCl treatment for up to 3 days, the control and ST-8 or ST-4 plants accumulated similar levels of $Na^+$ (Fig. 4A). However, the $Na^+$ content in control plants increased markedly after 5 days of NaCl treatment, whereas only small increases in the $Na^+$ content were found in ST-8 and ST-4 plants.

$Na^+$ is transported from root to shoot by the transpirational stream in the xylem. To determine if the reduced level of $Na^+$ accumulation in transgenic plants was due to decreased $Na^+$ transport through the xylem, xylem sap was collected from control and transgenic plants. Without NaCl treatment, the $Na^+$ content in the xylem sap of ST-8 and ST-4 plants was not significantly different from that of control plants (Fig. 4B). After 1 day of 100 mM NaCl treatment, the $Na^+$ concentrations in the xylem sap of both *SOS1*-overexpressing and control plants were dramatically increased, but the level was relatively lower for ST-8 and ST-4 plants (Fig. 4B). The $Na^+$ content in the xylem fluid of ST-8 and ST-4 plants was ~22% and 19% lower, respectively, than that of control plants in response to 1 day of 100 mM NaCl treatment. These results suggest that overexpression of *SOS1* may increase $Na^+$ retrieval from the xylem, thereby limiting $Na^+$ accumulation in the shoot.

Enhanced $Na^+$ efflux at the cellular level. We regenerated callus cultures from homozygous ST-8 and ST-4 transgenic plants to determine the effect of *SOS1* overexpression at the cellular level. Overexpression of *SOS1* conferred on the callus tissues increased tolerance to NaCl stress compared with that of wild-type control, whereas the *sos1* mutant callus was highly sensitive to salt stress (Fig. 5A). Measurements of fresh weight gains indicated less growth reduction in the transgenic calli than in wild-type calli (Fig. 5B). To determine if the improved tolerance was caused by reduced $Na^+$ accumulation, the $Na^+$ content in ST-8 and wild-type control calli was measured. As shown in Figure 5C, the $Na^+$ content showed no significant difference between ST-8 and control calli without NaCl treatment or after 1 day of 100 mM NaCl treatment. However, the ST-8 calli accumulated less $Na^+$ than the control after 3 and 5 days of NaCl treatment (Fig. 5C). Compared with the control, the $Na^+$ concentration in ST-8 calli was ~17% less after 3 days of NaCl treatment, and ~23% less after 5 days of NaCl treatment. These results suggest that *SOS1* overexpression confers salt tolerance to callus tissues by increasing $Na^+$ efflux and thereby reducing $Na^+$ accumulation in the cells.

## Discussion

Plant salt tolerance is a complex trait that involves multiple physiological and biochemical mechanisms and numerous genes. Accordingly, different strategies need to be tested experimentally to genetically improve salt tolerance of plants. Ultimately, the different strategies should be integrated, and genes representing distinctive approaches combined to substantially increase plant salt tolerance.

Theoretically, three mechanisms can be used to prevent excess $Na^+$ accumulation in the plant symplast. First, $Na^+$ entry into plant cells may be reduced once $Na^+$ influx transporter genes are identified in plants. Second, $Na^+$ that enters the cells can be transported and stored in the vacuoles. Overexpression of the vacuolar $Na^+/H^+$ transporter AtNHX1 was recently shown to confer salt tolerance in

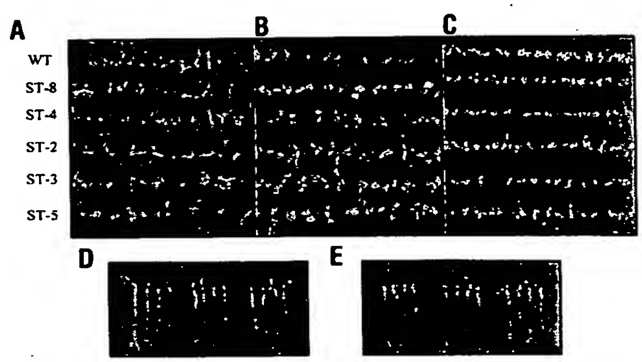


**Figure 3.** Enchanced salt tolerance of *SOS1*-overexpressing plants during early seedling development. (A) Seed germination in MS medium without NaCl. (B) Seed germination in MS medium plus 50 mM NaCl. (C) Seed germination in MS medium plus 150 mM NaCl. (D) Representative seedlings from A. (E) Representative seedlings from B. Seeds were surface-sterilized and plated onto the medium. After 3 d of stratification at 4°C, plates were transferred to 22°C in a culture chamber, and the pictures were taken a week later.

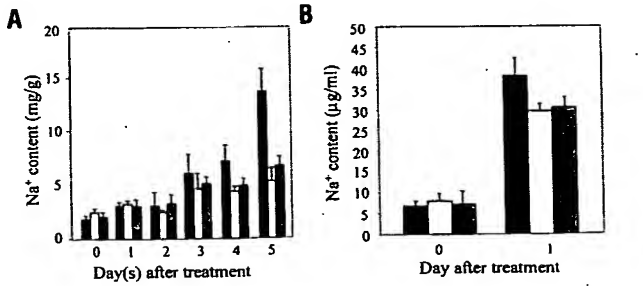


**Figure 4.** Reduced $Na^+$ accumulation in plants overexpressing *SOS1*. (A) $Na^+$ content in control and *35S:SOS1* transgenic plants ($n = 3$). (B) $Na^+$ content in the xylem sap of control and *35S:SOS1* plants ($n = 6$). Black solid bar, control plants; open bar, ST-8 transgenic line; gray solid bar, ST-4 transgenic line. Error bars represent s.d.

*A. thaliana* and tomato[22,23], suggesting the utility of this vacuolar compartmentation strategy. One potential limitation of the strategy, however, is that some critical cells like the root meristematic cells do not have large vacuoles. In fact, the *AtNHX1* gene is not expressed in the root meristem, and this tissue seems to rely on *SOS1* to keep out sodium[26]. Third, $Na^+$ in the cytoplasm can be exported back to external medium or the apoplast via plasma membrane $Na^+/H^+$ antiporters. In the fission yeast *Schizosaccharomyces pombe*, loss-of-function mutations in the plasma membrane $Na^+/H^+$ antiporter gene *SOD2* decrease salt tolerance whereas overexpression of *SOD2* substantially increases salt tolerance[28]. Very recently, overexpression of a plasma membrane $Na^+/H^+$ antiporter gene in a freshwater cyanobacterium, a photosynthetic organism, was shown to so dramatically enhance the salt tolerance that the transgenic cyanobacterium was able to grow in seawater[29]. The results reported here show that this $Na^+$ export strategy works to increase salt tolerance in plants.

In plants, the function of the plasma membrane transporter *SOS1* is complicated by the multiple tissues and root-shoot coordination. We have shown recently that *SOS1* is strongly expressed in parenchyma cells at the xylem-symplast boundary[26]. Under severe salt stress, this transporter seems to function in retrieving $Na^+$ from the xylem to prevent excess $Na^+$ accumulation in the shoot[26]. Although the CaMV 35S promoter is often considered constitutive, its activity is predominantly in the vasculature[30–32]. Therefore, the overexpression of *SOS1* in the xylem parenchyma cells may enhance the capacity to retrieve $Na^+$ from the transpirational stream in the *SOS1*-overexpressing transgenic plants. This notion is supported by our finding that *35S:SOS1* plants accumulated less $Na^+$ in the xylem and in the shoot (Fig. 4). It is also possible that in the *SOS1*-overexpressing transgenic plants, root epidermal cells may express *SOS1* and acquire the ability to export $Na^+$ to the soil solution. For osmotic balance, the transgenic plants are expected to have enhanced accumulation of other solutes in the vacuole and cytosol to compensate for the increased $Na^+$ export from cells.

In this study we have found that the *SOS1* transcript in the transgenic lines, although driven by the strong constitutive CaMV 35S promoter, is present at only a slightly higher level than in the wild type under normal growth conditions. However, the level is much higher under salt stress compared with wild type. This result suggests that the *SOS1* transcript is unstable in the absence of salt stress and that salt stress causes a posttranscriptional stabilization of the transcript. Posttranscriptional control of transcript accumulation is an important mechanism for gene regulation under stress, and has been seen previously for some salt-stress regulated genes[33,34] and for some abscisic acid– and water stress–regulated genes[35–37]. It would be interesting to identify the factor or factors that mediate *SOS1* transcript stabilization under salt stress.

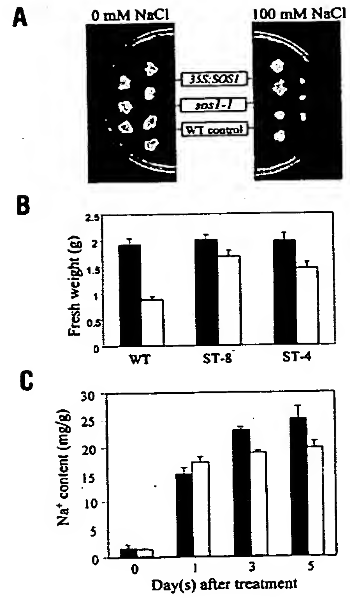


**Figure 5.** Calli overexpressing *SOS1* are more tolerant of NaCl. (A) Salt-tolerance test of calli. The calli were induced in MS medium with 1 mg/L 2,4-dichlorophenoxyacetic acid and 0.2 mg/L 6-benzylaminopurine. Calli were transferred to the same medium but supplemented with 100 mM NaCl and pictures were taken after 3 weeks of NaCl treatment. (B) Fresh weight of calli in response to NaCl treatment. Calli of similar sizes (about 0.12 g fresh weight) were chosen for the test of growth on medium with or without NaCl. Values are given as the means of 30 calli. Solid bar, 0 mM NaCl; open bar, 100 mM NaCl. (C) $Na^+$ content of *35S:SOS1* transgenic calli and wild-type calli ($n = 6$). Solid bar, wild type; open bar, ST-8. Throughout, error bars represent s.d.

## Experimental protocol

**Overexpression of *SOS1* in transgenic plants.** *SOS1* cDNA containing the complete open reading frame was obtained as previously described[25]. The cDNA was subcloned into pIG121-Hm by replacing the *GUS* coding region between the *Xba*I and *Sac*I sites, resulting in a construct for overexpression of *SOS1* under the control of the CaMV 35S promoter in plants. The construct was introduced into *Agrobacterium tumefaciens* strain GV 3101 by electroporation. *A. thaliana* (Columbia ecotype) wild-type plants were transformed by the vacuum infiltration method[38]. Transgenic plants harboring *35S:SOS1* were screened on MS agar medium (JRH Biosciences, Lenexa, KS) containing 40 mg/l kanamycin and the presence and integrity of the transgene were further confirmed by PCR amplification using primers specific for the 35S promoter and *SOS1* cDNA.

**RNA gel blot.** *A. thaliana* seedlings were grown on MS agar medium under continuous light[39]. For salt treatment, 10-d-old seedlings were transferred onto Whatman filter paper soaked with 100 mM or 200 mM NaCl and treated for 12 h. For the control treatment without NaCl, seedlings were transferred to filter paper soaked with MS solution only. Total RNA isolation and northern analysis were performed as described[40].

**Salt-stress tolerance tests.** $T_2$ overexpression transgenic plants and control plants with vector only were screened from kanamycin medium. The kanamycin-resistant plants were transferred to MS medium without kanamycin and allowed to grow for one week. Plants were then transferred to 6 cm pots filled with soil and cultured for another week. All plants were grown in soil under a long-day cycle (16 h light, 8 h dark) and were watered from below to field capacity with a diluted nutrient solution 0.125× MS salts in water as needed. After transplantation to soil for one week, this solution was supplemented with NaCl. The supplementations consisted of four increasingly higher concentrations (50 mM, 100 mM, 150 mM, and 200 mM) of NaCl. The plants were treated for 4 d at each concentration, for a total of 16

d. On the 16th day representative plants were chosen and photographed. For the plant survival test, homozygous transgenic plants with four true leaves were transferred to MS agar medium containing 200 mM NaCl and cultured for 5 d. The treated plants were then transferred to soil under normal growth conditions for three weeks. Plants that survived and continued to grow were counted. Relative root growth in response to salt stress was measured as described previously[41]. Seeds were plated onto MS agar medium containing different concentrations of NaCl for the germination test. Callus cultures from transgenic plants, wild-type plants, and *sos1* mutant were induced as described before[39]. For salt-tolerance tests, calli of similar size were selected and cultured on callus-induction medium with and without 100 mM NaCl. After three weeks of treatments, the fresh weight of calli was measured and callus pictures were taken.

**Measurement of $Na^+$ content.** *35S:SOS1* and vector-transformed plants grown in soil for three weeks were treated with 0.05× MS salts plus 100 mM NaCl for the indicated number of days. Calli were transferred to callus induction medium plus 100 mM NaCl for the indicated number of days. For xylem sap collection, plants were grown in soil to the stage of bolting and treated with 0.05× MS salts plus 100 mM NaCl for 1 d[26,42]. $Na^+$ content was measured as previously described[26].

**Chlorophyll fluorescence measurement and other methods.** Chlorophyll fluorescence was measured using an OS1-FL modulated fluorometer (Opti Sciences, Tyngsboro, MA). Fluorescence parameters were defined as follows: Fs, steady-state fluorescence under given environmental conditions; Fms, maximal fluorescence under steady state. The yield of quantum efficiency was calculated as $Y = (Fms - Fs)/Fms$[43]. Chlorophyll was extracted using 80% acetone and determined by the method of MacKinney[44]. Protein content was determined using a Bio-Rad protein assay kit.

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*Competing interests statement*
*The authors declare that they have no competing financial interests.*

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